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Inhibition of degranulation of human polymorphonuclear leukocytes by complement factor D

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Abstract A degranulation inhibiting protein could be isolated from human plasma ultrafiltrate by a three-step purification method including ion-exchange chromatography, gelfiltration and affinity-chromatography. The protein was identified as complement factor D by means of sequence analysis. Its degranulation inhibiting activity was determined with regard to its effect on the FNLPNTL-induced lactoferrin secretion of human polymorphonuclear leukocytes. Complement factor D caused a dose-dependent decrease of the FNLPNTL-stimulated lactoferrin degranulation down to 34% of stimulated controls.

Key words: Complement factor D; Adipsin; Renal failure; Neutrophil; Degranulation

1. Introduction

Patients undergoing haemodialysis as a consequence of renal failure often are susceptible to bacterial infections. This effect can be related with a restriction of leukocyte-functions [1] as chemotaxis, oxidative burst, degranulation and glucose-uptake. These dysfunctions may be attributed to physical damage of the cells during dialysis or to inhibitory factors, which should be found in plasma of these patients. An unlimited biological source of these proteins is the plasma ultrafiltrate obtained during haemodialysis. This fluid has been shown to contain some granulocyte inhibiting proteins. One of them was already described by Hörl et al. [2] and another has been recently found by Tschesche et al. [3]. In this article we report on a degranulation inhibiting protein isolated from human plasma ultrafiltrate and identified as complement factor D of the alternative complement pathway.

Complement factor D, identical to human Adipsin [4], is a serine protease responsible for cleaving complement factor B into the fragments Ba and Bb [5]. The latter factor forms in combination with C3b the C3/C5 convertase of the alternative complement pathway [6]. Factor D has a molecular weight of 24 kDa. consists of one polypeptide chain and is inactivated by diisopropyl-fluorophosphate [7]. In human plasma it is found to occur as an active enzyme, not as a zymogen [5]. Serum of healthy donors contains factor D in low concentrations (1–2 μg/ml), whereas this level can be increased ten-fold in serum of dialysis-patients suffering from chronic renal failure [8]. These plasma samples show an increase in alternative pathway activity [9] and a decreased activity of complement-mediated immune complex clearance [10]. Factor D can be isolated from various sources such as serum, peritoneal fluid and urine [6,11,12]. No influence of factor D on leukocyte functions has been described previously.

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Abbreviations BCA, bicinchoninic acid; DPBS, Dulbecco's phosphate-buffered saline; ELISA, enzyme linked immunosorbent assay; FNLPNTL, formyl-norleucyl-leucyl-phenylalanyl-norleucyl-tyrosyl-leucin; PBS, phosphate-buffered saline; PMNL, polymorphonuclear leukocytes; PTH, phenylthiohydantoin; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

2. Materials and methods

2.1 Materials

Plasma ultrafiltrates were obtained from University Freiburg, Department of Medicine, Dialysis Unit. An ultrafiltration cell was purchased from Amicon (Beverly, USA). CM Sephadex C50 and Sephacryl S-100 were supplied by Pharmacia (Uppsala, Sweden). Fresh blood was supplied by Prof. Kleesiek, Herzzentrum Bad Oeynhausen (Bad Oeynhausen, Germany) Ultravist 370 was a donation from Schering (Berlin, Germany). Methocel, Histopaque 1077 and anti-human lactoferrin were obtained from Sigma (München, Germany). Culture dishes and immunoplates were purchased from Nunc (Roskilde, Denmark). BCA protein assay kit was supplied by Pierce (Illinois, USA).

2.2 Purification of complement factor D from plasma ultrafiltrates

The following purification steps were carried out at 4°C. Plasma ultrafiltrates of different patients were concentrated 500-fold using an Amicon ultrafiltration cell with a YM3 membrane. The concentrated ultrafiltrate was applied to a column (40 × 3 cm) of CM Sephadex C50, previously equilibrated with sodium phosphate buffer, pH 6.0 (0.05 M NaH_2PO_4, 0.01 M EDTA, 0.2 M NaCl). The column was eluted with 500 ml of a linear NaCl gradient (0.2–2.0 M) in the same buffer at a flow rate of 60 ml/h. Fractions belonging to the same elution peak were pooled and concentrated. The sample containing factor D was concentrated as described above, applied to a Sephacryl S-100-column (80 × 3 cm) and eluted with Tris-buffer, pH 8.0 (0.02 M Tris, 0.005 M $CaCl_2$, 0 5 M NaCl) at a constant flow rate of 12 ml/h The fractions that corresponded to a molecular weight of 14–30 kDa were pooled and concentrated One fraction showing degranulation inhibiting activity was applied to a column (5 × 1.5 cm) of red Sepharose (prepared as described by Böhme [13]), previously equilibrated with 20 mM Tris, 5 mM $CaCl_2$, pH 8.3, and eluted using 120 ml of a linear NaCl gradient (0–1 M) in the same buffer (flow rate. 90 ml/h). The fractions were pooled according to their elution peaks, concentrated and analysed by electrophoresis and PMNL degranulation tests.

2.3. Electrophoresis

To control the progress of purification, the different fractions were analysed by SDS-PAGE performed as described by Weber and Osborn [14] using 10.3% polyacrylamide gel. The silver staining of the gel was conducted according to Damerval [15].

2.4. Amino acid sequencing

Automated Edman degradation for determination of the NH_2-terminal amino acid sequence was performed on a microsequencer (Model 810, Knauer, Berlin) with on-line PTH-analysis coupled to a Knauer HPLC-system equipped with an Applied Biosystems PTH-C18 column (220 × 2 1 mm).

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2.5 Determination of protein concentration

Protein concentrations were determined by means of a commercial BCA protein assay kit.

2.6. Preparation of polymorphonuclear leukocytes

Human PMNL were isolated from fresh blood of healthy donors by sedimentation of erythrocytes on a mixture of Ultravist 370 (18.2 ml/100 ml) and Methocel (1.4 g/100 ml H_2O) followed by centrifugation over Histopaque 1077 and hypotonic lysis of residual erythrocytes. The cells were suspended in DPBS-buffer (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% glucose).

2.7. PMNL degranulation test

Degranulation was examined using 10^6 leukocytes in a final volume of 0.5 ml DPBS-buffer. Protein samples, previously dialysed against PBS-buffer (DPBS-buffer without glucose), were preincubated at 37°C in culture-dishes (Nunc, Roskilde, Denmark), then the prewarmed cells were added and the assay was incubated for 10 min at 37°C. Addition of 10^{-7} M FNLPNTL (Sigma), further incubation at 37°C for 10 min and centrifugation of the supernatant followed.

2.8. ELISA for lactoferrin

The secretion of lactoferrin by PMNL was quantified using a specific sandwich-ELISA described by Schettler [16] and antibodies were prepared according to Bergmann [17].

3. Results and discussion

3.1. Isolation of factor D from plasma ultrafiltrate

Complement factor D as a degranulation inhibiting protein was isolated from plasma ultrafiltrates by means of ion-exchange-chromatography, gel permeation chromatography and affinity-chromatography. Its homogeneity was shown by SDS/PAGE (Fig. 1) and its molecular weight was found to be approximately 24 kDa.

3.2. Identification of factor D

The isolated protein was identified as complement factor D by amino acid sequence determination. The first 30 amino acid residues of the protein were determined to be ILGGREAEAHARPYMASVQLNGAHLCCGVL and found to be identical with the corresponding fragment of human complement factor D [18].

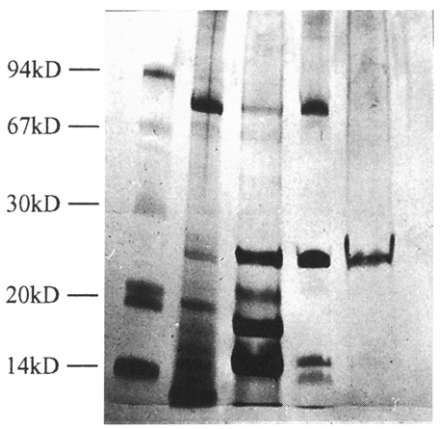

Fig 1. Progress in purification of factor D from human haemodialysis ultrafiltrate SDS-PAGE, proteins visualised by silver staining. Molecular weight marker (lane 1), concentrated ultrafiltrate (lane 2), eluate from CM Sephadex C50 (lane 3), eluate from Sephacryl S-100 (lane 4), isolated complement factor D (lane 6).

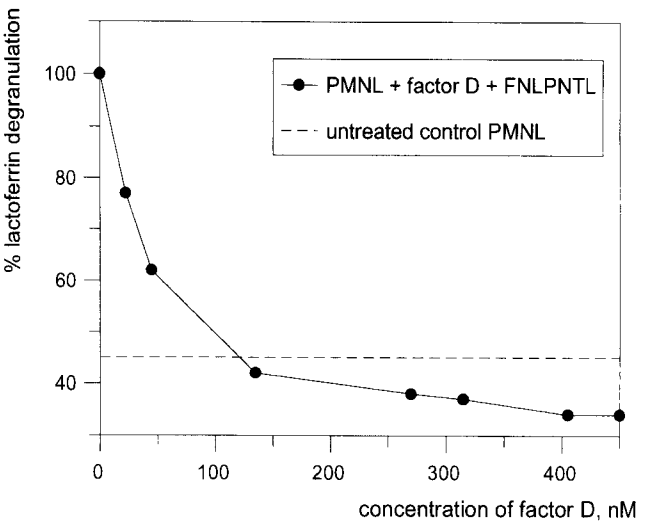

Fig. 2. Influence of complement factor D on FNLPNTL-stimulated lactoferrin degranulation of PMNL. Lactoferrin release of cells not treated with factor D but stimulated with FNLPNTL was set to 100%. The data shown represent the results of five separate studies.

3.3. Inhibition of PMNL-degranulation by complement factor D

The inhibitory activity of factor D on the granule secretion of human polymorphonuclear neutrophil leukocytes was examined with regard to its effect on PMNL stimulated with the synthetic peptide FNLPNTL. All experiments were conducted in a phosphate buffered saline medium containing 0.1% glucose at a temperature of 37°C. The PMNL were pretreated with complement factor D for 10 min and stimulated with FNLPNTL (10^{-7} M) for a further 10 min. The lactoferrin secretion of cells treated only with the formylpeptide was set to 100%. Spontaneous degranulation of untreated cells tested under the same conditions was found to be 45% of this value. Pretreatment with factor D caused an inhibition of degranulation dependent on its concentration. Fig. 2 shows the corresponding dose-response curve. Preincubation with factor D in concentrations from 22.5 nM to 450 nM diminished the activating effect of 10^{-7} M FNLPNTL on the lactoferrin secretion of PMNL. Addition of factor D in concentrations from 135 nM to 450 nM decreased the extent of degranulation below the level of spontaneous degranulation.

The concentration of complement factor D in serum of healthy human donors was found to be 40–80 nM [8]. In this range factor D lowered the FNLPNTL-induced lactoferrin-degranulation of PMNL from 100% to 55%–65% in our experiments. These results indicate, that complement factor D might regulate leukocyte enzyme release in healthy human blood as a counterpart of well known PMNL-stimulating agents such as interleukin-8, tumour necrosis factor and leukotriene B_4.

Patients suffering from end-stage renal failure can show a tenfold increase of factor D plasma-concentration up to 400–800 nM [8]. This causes an increase in activity of the alternative complement pathway [9] but also a decrease in complement-mediated immune complex clearance as described by Inagi [10]. Because of these pathophysiological effects, complement factor D has been identified as an important factor of dialysis-associated bioincompatibility [19]. Some membrane materials such as polyacrylonitril [20], polystyrol sulphonate [21] and polymethylmethacrylate [22] were found to adsorb factor D. Its removal

during haemodialysis might be beneficial for the immune system of uraemic patients.

In our experiments factor D applied in concentrations of 200–450 nM caused a decrease of FNLPNTL-induced lactoferrin-release down to 34%, while totally unstimulated cells showed a spontaneous lactoferrin-degranulation of 43%. This new physiological effect of complement factor D can contribute to a reduced host defence activity in patients with renal failure and high serum concentrations of factor D.

Human complement factor D obviously has three different regulatory effects on the functions of the immune system. It acts (I) as an activator of the alternative complement pathway, (II) as an inhibitor of immune complex clearance and (III) as an inhibitor of premature leukocyte degranulation. The signal transduction pathway responsible for these effects will be an object of further studies.

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